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CD4 T cell priming in dendritic cell-deficient mice

Paola Castiglioni[1], Christina Lu[1], David Lo[2], Michael Croft[3], Pierre Langlade-Demoyen[4], Maurizio Zanetti[1] and Mara Gerloni[1]

[1]Department of Medicine and Cancer Center, University of California San Diego, 9500 Gilman Drive, La Jolla CA 92093-0837, USA
[2]Digital Gene Technologies, La Jolla, CA 92037, USA
[3]Division of Immunochemistry, La Jolla Institute for Allergy and Immunology, La Jolla, CA 92121, USA
[4]Laboratoire d'Immunite Cellulaire Anti-Virale, Institut Pasteur, 75724 Paris, France

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Abstract

Bone marrow (BM) chimeras (BMC) generated from mice carrying a null (–/–) mutation in the *rel*B gene of the NF-κB family represent an ideal model for *in vivo* studies on the role of dendritic cells (DC) in the adaptive immune response. The spleen and lymph nodes (LN) of *rel*B$^{-/-}$ BMC contain a small number of residual DC, mainly CD8α$^+$, that fail to up-regulate MHC class II and co-stimulatory molecules after stimulation *in vitro*. Moreover, residual spleen DC of *rel*B$^{-/-}$ BMC have a 4-fold decrease in the ability to uptake and process soluble model antigen, ovalbumin (OVA), and failed to prime CD4 and CD8 T cells *in vitro* and *in vivo*. In addition, they also failed to present OVA peptide to OT-II transgenic T lymphocytes at a normal 1:10 (stimulator:responder) cell ratio. In spite of these multiple DC defects, *rel*B$^{-/-}$ BMC immunized with plasmid DNA targeted to the spleen as the site of immune induction develop a specific CD4$^+$ T cell response comparable to that of *rel*B competent mice. These data demonstrate that CD4$^+$ T cells can be primed in the absence of functional DC and suggest that *rel*B may gauge the T cell response *in vivo*.

Introduction

During the past 15 years it has become apparent that dendritic cells (DC) play a key role in the initiation of the adaptive immune response (1). Their extraordinary capacity to activate T cells is explained in part by their high expression of MHC class II and co-stimulatory molecules, and in part by their localization within secondary lymphoid organs (the marginal zone and T cell-rich areas) which gives them a strategic advantage for the interception and presentation of antigen (2).

DC patrol the organism by virtue of a widespread distribution in lymphoid and non-lymphoid tissues, and by their ability to promptly migrate from non-lymphoid tissues to draining lymph nodes (LN) (3). Migration together with a rapid transition from immature to mature phenotype under phlogogenic stimulation (microbial and inflammatory products) (4) render DC the ideal antigen-presenting cells (APC) for T cell responses (5). Upon phlogogenic stimuli DC up-regulate and stabilize expression of MHC class II molecules and co-stimulatory molecules (CD40, CD80 and CD86), and produce IL-12 for a brief time to enable T_h1 responses (6). Activated DC control the emerging immune response through dynamic and molecular adaptations possibly unique among immune cells. However, in spite of abundant *in vitro* and *ex vivo* (7–11) evidence, the extent to which DC play a role *in vivo* has been probed only in a few instances (12,13) and no studies exist on their role during systemic immunity initiating in the spleen as the site of immune induction.

The spleen is the largest secondary lymphoid organ and the site where adaptive immunity likely develops in response to antigens trapped from the blood stream. Numerous antigens fall in this category, including those associated with infections transmitted by arthropods (bacteria, viruses and protozoa), blood transfusion, tumor cells, certain bacterial infections (e.g. pneumococcal bacteremia), virus-infected cells and ecotropic viruses associated with autoimmune diseases. There exists enough suggestive evidence that in mammals the spleen is not only an organ with hemocatheretic functions, but also a site of primary importance in the development of the adaptive immune response.

To probe the role of splenic DC in CD4 T cell priming we used mice that lack functional bone marrow (BM)-derived DC as a result of a null (–/–) mutation in the *rel*B gene which codes for a component of the NF-κB complex (14). Homozygous

Correspondence to: M. Zanetti; E-mail: mzanetti@ucsd.edu

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relB$^{-/-}$ mice have an atrophic thymic medulla, possess no LN and lack BM-derived DC (15). However, homozygous *relB*$^{-/-}$ mice possess CD8α^{+} lymphoid DC in the spleen (16). BM chimeras (BMC) generated by transferring homozygous $^{-/-}$ *relB* BM cells into lethally irradiated (1100 rad) hemizygous (+/–) *relB* recipients carry the same DC defect as *relB*$^{-/-}$ mice, but have a longer lifespan (14). In contrast to the severe deficit in DC, B cells are apparently normal. Experiments *in vitro* show that B lymphocytes of *relB*$^{-/-}$ mice proliferate to lipopolysaccharide (LPS) (our unpublished results), undergo isotype switch (17) and express normal levels of chemokine receptors (18). Experiments *in vivo* show that following immunization with virus or DNA *relB*$^{-/-}$ BMC produce antibodies and form germinal centers (14,19). Thus, *relB*$^{-/-}$ BMC constitute an ideal animal model to test the role of spleen DC in the generation of a T cell response.

In light of the foregoing we decided to study the ability of residual spleen DC in *relB*$^{-/-}$ BMC to prime T cell responses to a model antigen, ovalbumin (OVA), and compare these results with those obtained in response to DNA immunization where antigen is synthesized directly within the spleen. In this latter approach, termed somatic transgene immunization (20), immunity ensues as a result of direct intraspleen inoculation of plasmid DNA (21) coding for an immunoglobulin heavy (Ig H) chain gene. Previously, we showed that plasmid DNA is taken up by B lymphocytes that remain in the spleen (22) and, since the transgene is under the control of a B cell-specific promoter, B lymphocytes begin synthesis of transgenic Ig. By using Ig H chain genes coding for heterologous T cell epitopes in the complementarity determining regions of the variable domain (23) it has been possible to trigger specific CD4 (24) T cell responses. The process of *in vivo* transgenesis is also followed by secretion of sizeable amounts (up to 70 ng/ml) of transgenic Ig, and these are thought to reinforce immunity locally and spread it to secondary lymphoid organs systemically (24).

Methods

Mice and generation of BMC

C57BL/6 mice (8–10 weeks old) were purchased from the Jackson Laboratories (Bar Harbor, ME). Homozygous *relB* $^{-/-}$ mice were bred in the animal facility of the University of California San Diego. BMC were generated by injecting i.v. 5 × 10^{6} BM cells from *relB*$^{-/-}$ (15) or C57Bl/6 mice into lethally irradiated (1100 rad) hemizygous *relB*$^{+/-}$ or C57BL/6 recipients respectively. Mice were used 5–6 weeks after BM transfer. OT-I (25) and OT-II (26) TCR transgenic mice were the kind gift of Drs M. Croft and S. Schoenberger (La Jolla Institute for Allergy and Immunology).

Plasmid DNA, proteins and synthetic peptides

Plasmid γ1NV^{2}NA^{3} was engineered as described in (23). Plasmid DNA was purified using a Qiagen Megaprep kit (Qiagen, Chatsworth, CA). Purified plasmid was stored at –20°C until use. OVA (grade VII) was purchased from Sigma (St Louis, MO). FITC-conjugated OVA was purchased from Molecular Probes (Eugene, OR). Synthetic peptides NANPNVDPNANP, (–NVDP–), its control NANPNANPNANP, $(NANP)_3$, and OVA peptide ISQAVHAAHAEINEAGR (amino acids 323–339) were synthesized in the Peptide Chemistry Facility of the California Institute of Technology (Pasadena, CA).

DC isolation and culture

DC were isolated from spleens and LN of *relB*$^{-/-}$ BMC, C57Bl/6 BMC, *relB*$^{-/-}$ and C57Bl/6 mice respectively. Spleens and LN were digested with collagenase D (0.5 mg/ml) and DNase I (0.1 mg/ml) (Sigma) for 25 min at room temperature with frequent pipetting to break up fragments. The undigested fibrous material was removed by filtration through a nylon mesh. Samples of 10^{8} spleen cells and 10^{7} LN cells were cultured in six-well plates with IMDM (Gibco/BRL, Grand Island, NY) supplemented with 10% heat-inactivated FCS (HyClone, Logan, UT), recombinant mouse granulocyte macrophage colony stimulating factor (GM-CSF, 1000 U/ml; PharMingen, San Diego, CA) and recombinant mouse IL-4 (4 ng/ml; R & D Systems, Minneapolis, MN). Cultures were split and the medium was changed when necessary. On day 4 LPS (*Escherichia coli*, 10 μg/ml; Sigma) was added to cultures for an additional 24 h. For *in vitro* or *in vivo* experiments with OVA and OVA peptide, DC from spleens of *relB*$^{-/-}$ BMC, C57Bl/6 BMC and C57Bl6 mice were additionally purified using a discontinuous gradient. Briefly, after tissue digestion spleen cells were resuspended in PBS (Gibco/BRL) and OptiPreo (3:1 v/v; Accurate, Westbury, NY) giving a 1.085 g/ml solution. This suspension was over-layered with 5 ml of a 1.065 g/ml iodixanol solution and 3 ml of PBS, and centrifuged at 1600 r.p.m. for 15 min. The low-density fraction was collected and depleted of B cells using magnetic beads coated with a mAb against mouse CD19 (Miltenyi Biotech, Auburn, CA) followed by sorting on a MACS separation column (Miltenyi Biotech). CD19 $^{-}$ cells were additionally enriched in DC using CD11c MicroBeads (Miltenyi Biotech). Splenic DC were 85–90% pure.

T cell priming in vitro

The ability of residual spleen DC of *relB*$^{-/-}$ BMC to prime naive T lymphocytes was assessed in an *in vitro* culture system using naive C57Bl/6 CD4 and CD8 T lymphocytes and OVA (Sigma) as the antigen. C57Bl/6 spleen CD4 T lymphocytes were isolated using magnetic beads coated with a mAb against mouse CD4 (Miltenyi Biotech) followed by sorting on a MACS magnetic separation column (Miltenyi Biotech). The CD4 T cells obtained were 90% pure as determined by FACS analysis. CD8 T lymphocytes were enriched using magnetic beads coated with mAb against mouse CD4, CD19 and CD11c (Miltenyi Biotech). The CD8 T cell population was >60% pure. Purified spleen DC from *relB* $^{-/-}$ BMC, C57Bl/6 BMC or C57Bl/6 were plated on 96-well round-bottomed plates at 0–2 × 10^{4} cells/well and incubated overnight with OVA (0–500 μg/ml final concentration). Antigen-pulsed DC were irradiated (3000 rad) and syngeneic naive CD4 or CD8 T lymphocytes were added (5 × 10^{4} cells/well) (27). Supernatants were harvested on day 5 for cytokine detection and the proliferative response was measured by the uptake of [^{3}H]Thymidine added (1 μCi/well) on day 6 for 18–20 h. The assay was performed as detailed below. In additional experiments we pulsed purified spleen DC from *relB*$^{-/-}$ BMC and C57 Bl/6 BMC with $OVA_{323-339}$ peptide (1 μg/ml) and used

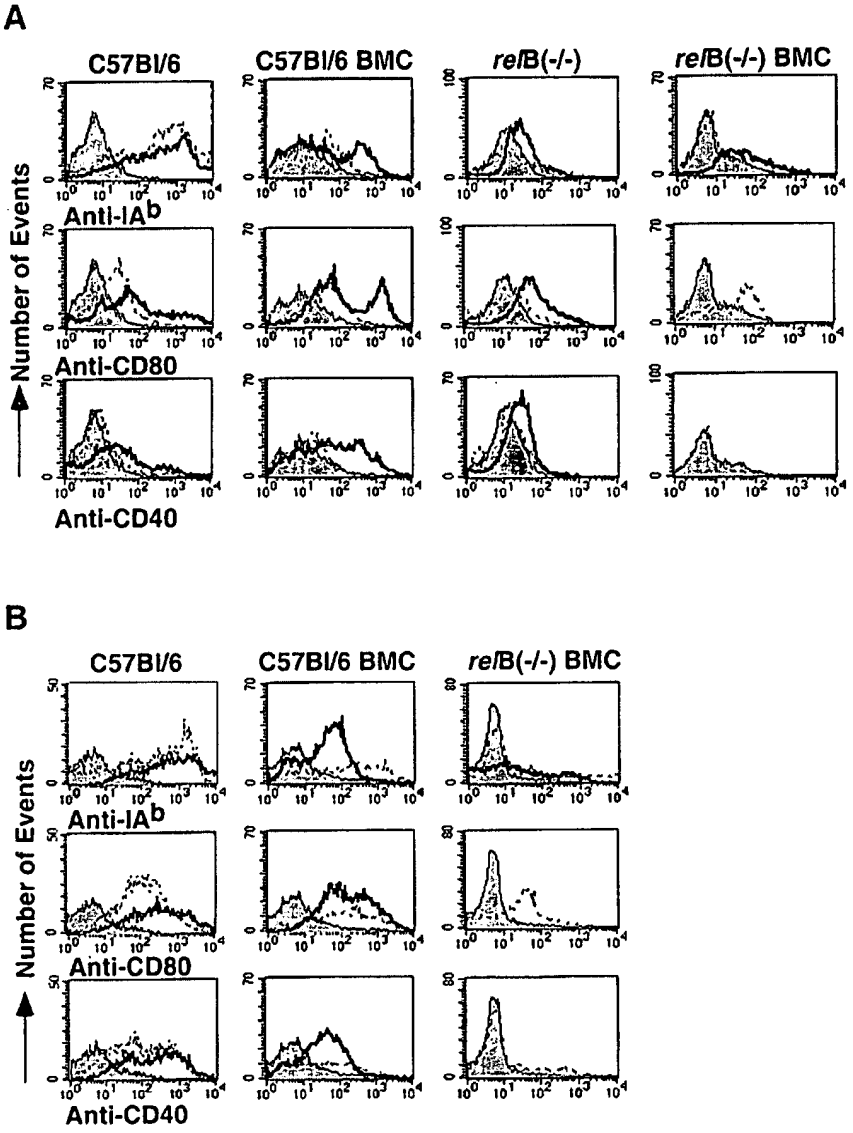

Fig. 1. Surface phenotype analysis and quantitation of DC isolated from spleens (A) of C57Bl/6, C57Bl/6 BMC, *rel*B $^{-/-}$ and *rel*B$^{-/-}$ BMC, and LN (B) of C57Bl/6, C57Bl/6 BMC and *rel*B$^{-/-}$ BMC. CD11c$^+$ cells were analyzed for the expression of MHC class II (I-A^b), CD80 and CD40 surface molecules. The results are shown as histograms with fluorescence intensity on the *x* -axis and cell number on the *y*-axis. Dotted lines represent *ex vivo* staining of spleen cells, whereas thick lines represent staining after 4 days in culture with GM-CSF, IL-4 and LPS which was added during the following 24 h as described in Methods. Solid histograms refer in each instance to the background (i.e. autofluorescence).

them (0–5 × 10^4 cells/well) to stimulate OT-II CD4 T lymphocytes (10^5 cell/well). OT-II splenocytes were depleted of APC and CD8 T cells using a cocktail of mAb and rabbit complement. The mAb were M5114 anti-I-A, CA4 anti-class II, RA3.6.B2 anti-B220, PK136 anti-NK, M1/70 anti-CD11b and 3.155 anti-CD8 (kindly provided by Stephen Schoenberger, La Jolla Institute for Allergy and Immunology).

In vivo immunization procedures

DNA immunization. Mice were inoculated intraspleenically with 100 μg of plasmid DNA in 50 μl of sterile saline solution as previously described (21).

Immunization with OVA. The ability of residual spleen DC in *rel*B$^{-/-}$ BMC to uptake and process OVA was tested by i.v. injection of soluble OVA (3 mg/mouse) dissolved in PBS. After 20 h DC from pools of two to four spleens were isolated as described above and cultured (0–6000/well) with 30,000 OVA-specific CD8 T cells from OT-I mice (>60% purity). Purified DC were also cultured (0–10^5/well) with 10^5 OVA-specific CD4 T cells from OT-II mice (> 50% purity). Culture medium contained murine recombinant IL-2 (50 U/ml). Supernatants for cytokine detection were harvested after 40 h and the T cell proliferation response measured as detailed below. PBS alone was injected in C57Bl/6 mice to control for

Table 1. DC in the spleen and LN of *rel*B$^{-/-}$ BMC

	C57Bl/6	C57Bl/6 BMC	*rel*B$^{-/-}$	*rel*B$^{-/-}$ BMC
Total DC per spleen[a]	1.1 ± 0.2 (1)	0.5 ± 0.2 (0.4)	0.5 ± 0.1 (0.3)	0.6 ± 0.4 (0.3)
Total DC after culture[b]	0.5 ± 0.1 (3)	1.6 ± 1.4 (6.4)	0.2 (1.25)[c]	0.06 (0.2)
Total DC in LN[d]	0.2 ± 0.1 (0.8)	0.4 (1.3)		0.15 (1.5)
Total DC after culture[e]	0.6 (24)	0.3 (27.3)		0.02 (2.8)

[a]Values represent the total number of DC (×10^6) (and percentage over the total number of cells) in the spleen *ex vivo*. Results are expressed as the mean ± SD of three to five mice.

[b]Values represent the total number of DC (×10^6) (and their percentage) harvested after *in vitro* culture of 10^8 spleen cells as described in Methods.

[c]Value refers to a pool of three to five mice.

[d]Values represent the total number of DC (×10^6) (and their percentage over total number of cells) in LN *ex vivo*. Eight to ten LN were harvested from each mouse. Results refer to a pool of three to five mice. In the case of C57Bl/6 mice the result refers to the mean ± SD of three mice.

[e]Values represent the total number of DC (×10^6) (and their percentage) harvested after *in vitro* culture of 10^7 LN cells as described in Methods.

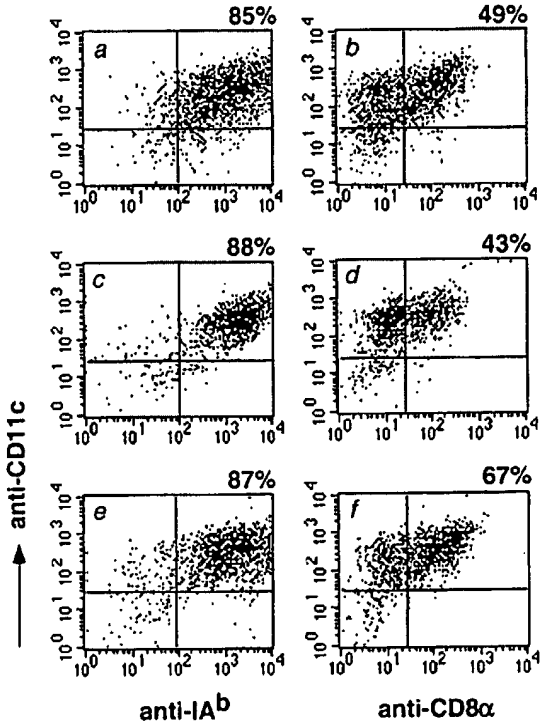

Fig. 2. Surface phenotype analysis of DC purified from spleen cell suspensions of C57Bl/6 (a and b), C57 Bl/6 BMC (c and d) and *rel*B$^{-/-}$ BMC (e and f). After purification DC were stained with an anti-CD11c, anti-MHC class II (I-A^b) and anti-CD8α mAb. The percentage of CD11c/MHC class II/CD8α-positive cells is shown in each panel. Results shown are representative of three to five independent purifications of pools of two to four mice in each instance.

the response of T cells to DC in the absence of OVA (background response). In uptake studies FITC-conjugated OVA was injected i.v. (2 mg/mouse). Spleen DC were isolated 1 h later from a pool of three or four spleens. Non-fluorescent native OVA was injected into control mice to provide the DC background for FITC labeling (28).

[^{3}H]Thymidine incorporation assay

Fourteen days after DNA inoculation mice were sacrificed and the spleens were removed to prepare single-cell suspensions. Red blood cells were eliminated with lysis buffer (Sigma). Lymphocytes were cultured (10^6 cells/ml) in RPMI 1640 medium (Irvine, Santa Ana, CA) supplemented with 7.5% FCS and 50 μM 2-mercaptoethanol, in the presence or absence of synthetic peptide –NVDP– or control peptide $(NANP)_3$ (50 μg/ml) in triplicate wells by incubation at 37°C in 10% CO_2 for 3 days. [^{3}H]Thymidine was added at 1 μCi/well and the cells were incubated for 16–18 h at 37°C. Cells were harvested onto glass fiber filter mats using a Tomtec cell harvester and the radioactivity was measured in a liquid scintillation counter (Betaplate; Wallac, Turku, Finland). Results are expressed as means ± SD of c.p.m. of triplicate cell cultures.

Detection of cytokines

Culture supernatants were harvested 40 h after initial seeding (or 5 days after, for *in vitro* T cell priming experiments) and were stored at –20°C. The supernatants from three separate triplicate cultures were pooled for each mouse. IL-2 activity was determined either through a bioassay utilizing the IL-2- and IL-4-dependent NK.3 cells in the presence of anti-IL-4 (purified from the 11B11 cell line; ATCC, Rockville, MD) as described previously (24) or using the OptEIA mouse IL-2 set (PharMingen, San Diego, CA) (Figs 3 and 5). IL-4 and IFN-γ were measured in the same culture supernatants by ELISA as described previously (24). Standard curves were constructed with purified IL-2, IL-4 and IFN-γ. Tests were performed in duplicate.

Phenotype analysis

The phenotyping of DC and their quantitation were performed on collagenase-treated spleen and LN cells *ex vivo* and after *in vitro* culture. Single-cell suspensions from *rel*B$^{-/-}$BMC, C57Bl/6 BMC, *rel*B$^{-/-}$ and C57Bl/6 were incubated with magnetic beads coated with a mAb against mouse CD11c (Miltenyi Biotech) and then sorted on a MACS separation column (Miltenyi Biotech). Cells selected on the basis of CD11c expression were then stained with biotin-conjugated mAb against mouse CD11c (clone HL3) (PharMingen). After

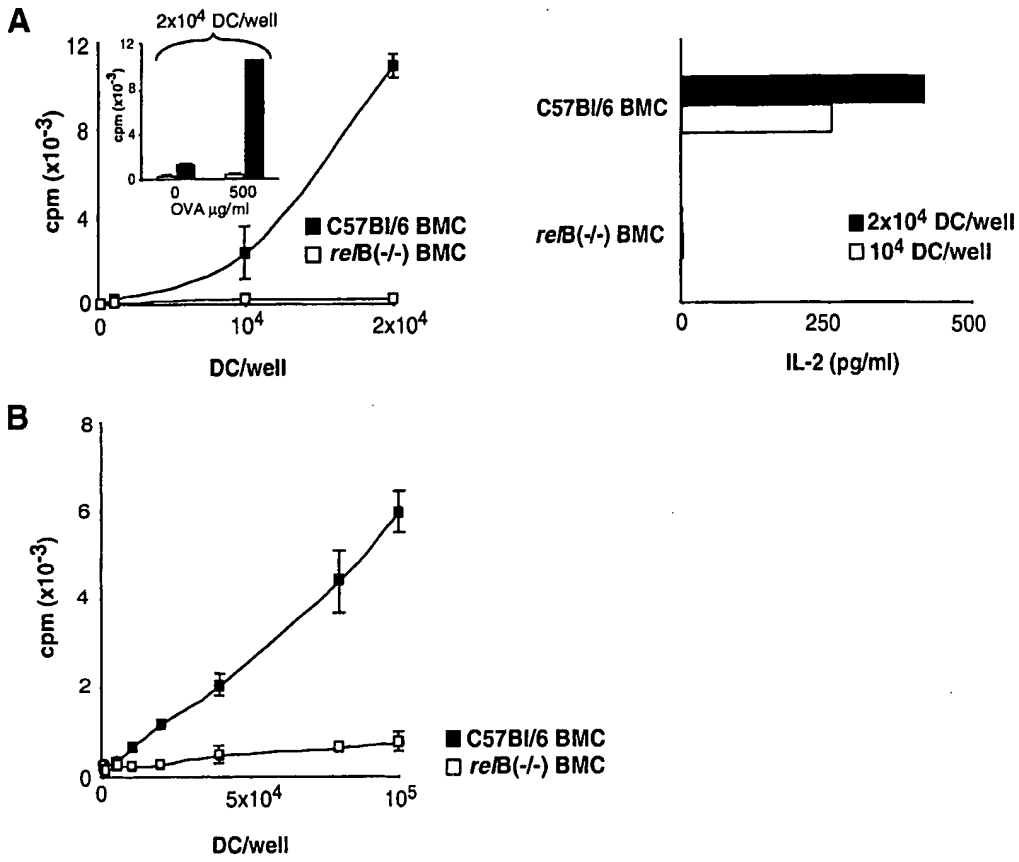

Fig. 3. Spleen DC of *relB* $^{-/-}$ BMC fail to prime CD4 T cell responses against OVA. (A) OVA-pulsed spleen DC from *relB*$^{-/-}$ BMC or C57Bl/6 BMC were used to prime naive CD4 T lymphocytes from the spleen of C57Bl/6 mice. CD4 T cells (5×10^4) were cultured with different concentrations of DC as indicated in each panel. The experiments shown were performed pulsing DC with OVA at 500 µg/ml. For IL-2 detection culture supernatants were harvested on day 5. [^{3}H]Thymidine incorporation was measured on day 6. The inset in the right panel shows that non-pulsed DC (2×10^4) do not elicit any proliferative response. (B) *relB*$^{-/-}$ BMC and C57Bl/6 BMC were injected i.v. with OVA (3 mg). At 20 h after injection, DC were purified from a pool of two to four spleens and cultured at different concentrations (0–10^5 cells/well) with 10^5 OT-II CD4 T lymphocytes. [^{3}H]thymidine incorporation was measured after 72 h. Proliferation assays were run in triplicate. Results are from a single experiment representative of two experiments with a total of five *relB*$^{-/-}$ BMC examined.

20–30 min at 4°C, cells were washed with PBS containing 0.5% BSA and 0.05% NaN_3, and stained with CyChrome–streptavidin and one of the following phycoerythrin-conjugated mAb: anti-I-A^b (clone AF6-120.1), anti-CD80 (clone 16-10A1), anti-CD40 (clone 3/23), anti-D^b (clone KH95) and anti-CD8 (clone 53-6.7) (PharMingen). Spleen DC from FITC–OVA-injected mice were isolated and double-stained with CD11c (clone HL3) mAb. Cells were analyzed by flow cytometry on a FACSCalibur (Becton Dickinson) and gates were set to select for viable DC. For DC quantitation cells were gated on CD11c/I-A^b double-positive cells.

Results

Phenotypic characterization of residual DC in relB$^{-/-}$ *BMC*

The phenotypic characteristics of residual DC in the spleen of *relB*$^{-/-}$ BMC were analyzed and compared with those of C57Bl/6 BMC, *relB*$^{-/-}$ and C57Bl/6 mice. Analyses were performed *ex vivo* (i.e. immediately after tissue harvest) and *in vitro* following culture with IL-4 (4 ng/ml) and GM-CSF (1000 U/ml) for 96 h plus LPS (10 µg/ml) during the last 24 h. FACS analyses were carried out on CD11c$^+$ cells double-stained for MHC class II (I-A^b), CD40 or CD80. Splenic DC from both homozygous *relB*$^{-/-}$ mice and *relB*$^{-/-}$ BMC (Fig. 1 A) have a much reduced expression of these cell surface molecules compared with C57Bl/6 mice or their BMC. An analysis of LN DC from *relB*$^{-/-}$ BMC showed a very similar defect (Fig. 1 B). Importantly, in *relB*$^{-/-}$ BMC and *relB*$^{-/-}$ mice up-regulation of MHC class II and CD40 or CD80 after *in vitro* culture and stimulation with LPS was minimal if any. In addition, even though CD11c$^+$ cells were counted *ex vivo* in both *relB*$^{-/-}$ BMC and *relB*$^{-/-}$ mice, no increase in number was noted after *in vitro* culture and LPS stimulation (Table 1). Consequently, lack of survival and maturation in culture made it impossible to profile CD40 and CD80 on these cells. Taken together these results suggest that

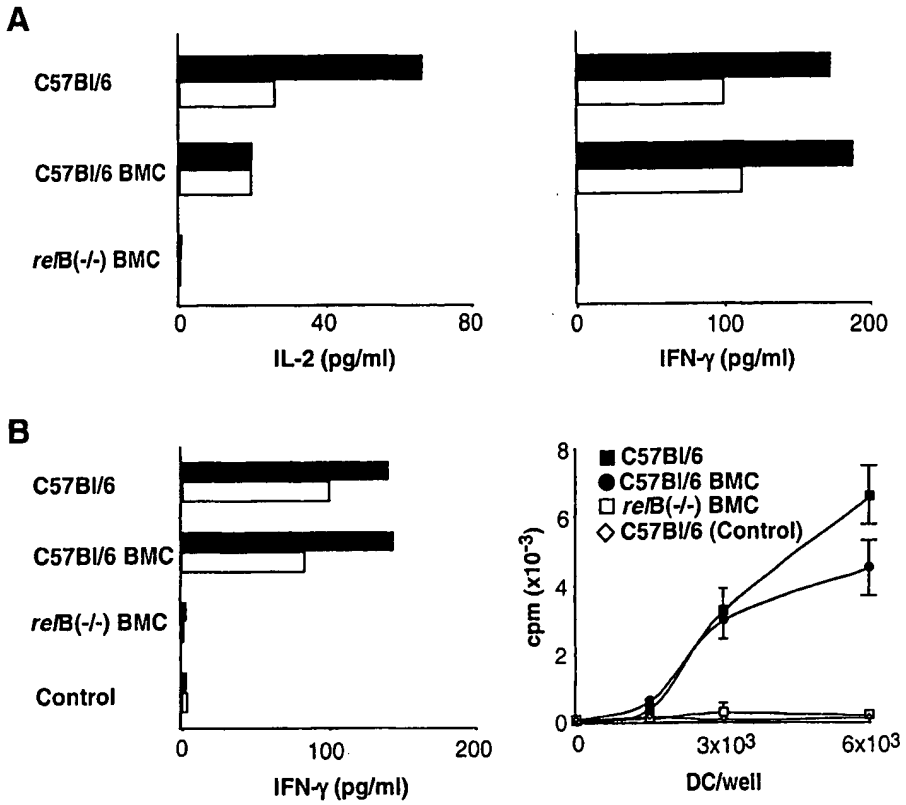

Fig. 4. Spleen DC of *relB*$^{-/-}$ BMC fail to prime CD8 T cell responses against OVA. (A) OVA-pulsed spleen DC from *relB*$^{-/-}$ BMC, C57Bl/6 BMC and C57Bl/6 were used to prime naive CD8 T lymphocytes from the spleen of C57Bl/6 mice. CD8 T cells (5×10^4) were cultured with different concentrations of DC (2×10^4, solid bars; 10^4, open bars). For IL-2 and IFN-γ detection culture supernatants were harvested on day 5. The experiments shown were performed pulsing DC with OVA at 500 μg/ml. (B) *relB*$^{-/-}$ BMC, C57Bl/6 BMC and C57Bl/6 were injected with OVA (3 mg) i.v. At 20 h after injection, DC were purified from a pool of two to four spleens and cultured at different concentrations (6×10^3, solid bars; 3×10^3, open bars) (left panel) with 3×10^4 OT-I CD8 T lymphocytes. The supernatants for IFN-γ detection were collected after 40 h and [^{3}H]thymidine incorporation was measured after 72 h. C57Bl/6 mice injected with PBS were used as a control. Cytokine detection assays were run in duplicate. Proliferation assays were run in triplicate.

CD11c$^+$ cells in *relB*$^{-/-}$ BMC have little if any constitutive expression of MHC class II and co-stimulatory molecules that fail to up-regulate after *in vitro* culture and LPS stimulation.

Splenic DC of relB$^{-/-}$ *BMC fail to prime T cells to soluble antigen*

We tested the ability of residual splenic DC of *relB*$^{-/-}$ BMC to process and present a model antigen (OVA) *in vitro* and *in vivo*. Both approaches required the isolation and enrichment (~85–90% purity) of DC from the spleen of *relB*$^{-/-}$ BMC, C57Bl/6 BMC and C57Bl/6 mice as controls (Fig. 2).

An initial *in vitro* experiment showed that purified spleen DC from *relB*$^{-/-}$ BMC pulsed overnight with OVA at 37°C failed to prime naive C57Bl/6 CD4 T lymphocytes. In fact, after a 6-day culture CD4 T cell proliferation occurred in cultures seeded with antigen-pulsed DC from C57Bl/6 BMC but not *relB*$^{-/-}$ BMC (Fig. 3A, left panel). A syngeneic mixed lymphocyte reaction can be ruled out in view of the fact that 2×10^4 DC/well in the absence of OVA did not induce any proliferation (see inset). Lack of CD4 T cell priming in cultures where *relB*$^{-/-}$ BMC DC served as the APC was mirrored by the absence of IL-2 production (Fig. 3A, right panel). Similarly, no T cell activation was found when antigen uptake and processing was first allowed to occur *in vivo*. To this end, *relB*$^{-/-}$ BMC, C57Bl/6 BMC and C57Bl/6 mice, were injected i.v. with OVA (3 mg/mouse) to enable antigen uptake by splenic DC (29). At 20 h after injection, DC were purified from the spleens and used as APC to activate naive, OVA-specific OT-II CD4 T lymphocytes (26). As shown in Fig. 3(B), CD4 T cell proliferation was observed in cultures seeded with DC from C57Bl/6 BMC, but not with DC from *relB*$^{-/-}$ BMC or from mice injected with PBS as controls (data no shown).

As an additional proof of the profound deficit in T cell priming by residual spleen DC of *relB*$^{-/-}$ BMC, new experiments were performed to explore the ability of OVA-pulsed DC to prime naive C57Bl/6 CD8 T cells. IL-2 and IFN-γ were detected only in cultures seeded with *in vitro*-pulsed DC purified from C57Bl/6 and C57Bl/6 BMC, but not *relB*$^{-/-}$ BMC (Fig. 4A). Similarly, in experiments in which uptake of OVA was performed *in vivo* followed by *in vitro* presentation to OVA-specific OT-I CD8 T lymphocytes (25), proliferation was observed only in cultures seeded with DC from C57Bl/6 and

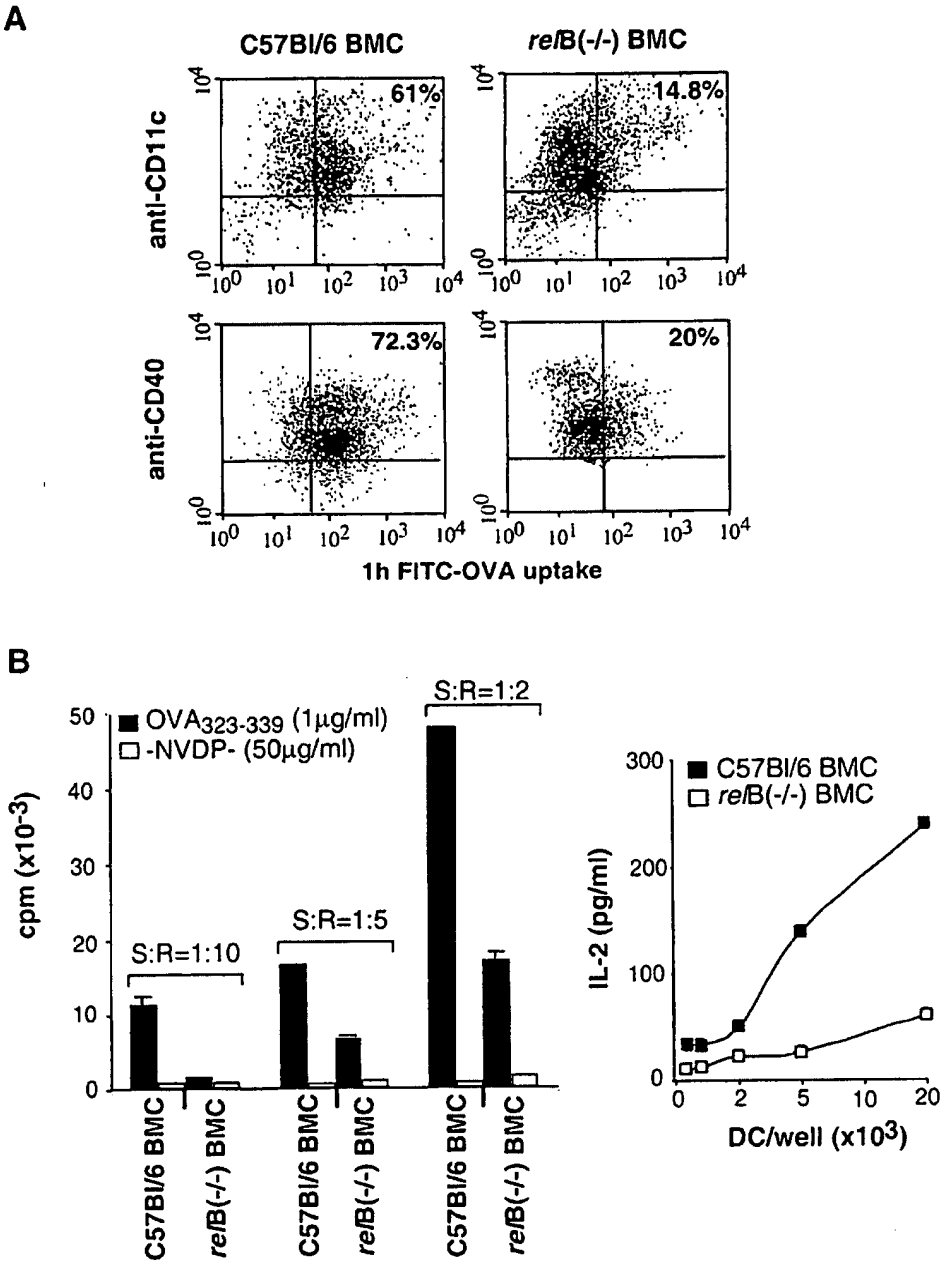

Fig. 5. Antigen uptake and presentation by residual spleen DC in *relB*$^{-/-}$ BMC. (A) Mice were injected i.v. with 2 mg of FITC-conjugated OVA. DC were isolated 1 h later from pools of three to four spleens, stained with an anti-CD11c and anti-CD40 mAb, and analyzed by flow cytometry. The percentage of FITC fluorescence within the DC is shown in each panel. (B) OT-II CD4 T lymphocytes were stimulated at different ratios with $OVA_{323-339}$ peptide pulsed spleen DC from *relB*$^{-/-}$ BMC and C57Bl/6 BMC. DC pulsed with –NVDP– peptide were used as control. [^{3}H]Thymidine incorporation (left panel) was measured after 72 h. Proliferation assays were run in triplicate. For IL-2 detection (right panel) supernatants were harvested after 40 h of culture. Tests were run in duplicate.

C57Bl/6 BMC, but not *relB*$^{-/-}$ BMC (Fig. 4B, right panel). No IFN-γ was detected in cultures seeded with DC from OVA-injected *relB*$^{-/-}$ BMC (Fig. 4B, left panel).

Collectively, these results show that residual spleen DC in *relB*$^{-/-}$ BMC are unable to prime T cell responses whether antigen uptake is allowed to occur *in vitro* or *in vivo*.

Deficit in antigen uptake and presentation by residual spleen DC in relB$^{-/-}$ *BMC*

Lack of T cell priming could be due to a deficit of antigen uptake and/or lack of presentation to T cells. To decide among these possibilities two experiments were performed. First, we

assessed the ability of splenic DC from *rel*B$^{-/-}$ BMC to capture FITC-conjugated soluble OVA. Within 1 h of injection spleen DC purified from C57Bl/6 mice and C56Bl/6 BMC showed extensive uptake of OVA (>60%) (Fig. 5 A). In contrast, OVA uptake by DC purified from *rel*B $^{-/-}$ BMC was much lower (14.8%). Thus, residual spleen DC in *rel*B$^{-/-}$ BMC have a much-reduced (4-fold) ability to capture soluble antigen relative to *rel*B competent mice. Second, we tested the ability of residual spleen DC from *rel*B$^{-/-}$ BMC to present the OVA $_{323-339}$ peptide to OT-II CD4 T lymphocytes (Fig. 5 B). Not surprisingly at a standard (1:10) stimulator (DC):responder (OT-II cells) ratio DC of *rel*B$^{-/-}$ BMC failed to induce T cell priming, whereas DC from control C57Bl/6 BMC showed a vigorous response. DC from *rel*B$^{-/-}$ BMC promoted T cell proliferation only when added in higher number/well that also altered the stimulator to responder ratio from 1:10 to 1:2. At this latter ratio DC from *rel*B$^{-/-}$ BMC induced a response that was ~3-fold lower than DC from C57Bl/6 BMC (left panel). The deficit in T cell priming was mirrored by a very low detection of IL-2 in cultures seeded with DC from *rel*B$^{-/-}$ BMC (right panel). Collectively, it appears that the defect in APC function by residual spleen DC in *rel*B$^{-/-}$ BMC is a complex one, including a much-reduced capacity to capture soluble antigen and an impaired ability to present processed antigen peptide.

CD4 T cell priming in the spleen of rel *B$^{-/-}$ BMC*

The above defects in APC function by spleen DC in *rel*B $^{-/-}$ BMC resulted in their inability to prime T cells against soluble antigens. Thus it became interesting to see if a CD4 T cell response could be induced *in vivo* using somatic transgene immunization (21) model DNA vaccination. Experiments were performed using a plasmid γ1NV^{2}NA^3 comprising an Ig H chain gene whose V domain is engineered to code for two dodecapeptides from the circumsporozoite protein of *Plasmodium falciparum* malaria parasite: the T_h cell determinant –NVDP– in the second complementarity determining region (CDR2) and the B cell epitope $(NANP)_3$ in the third complementarity determining region (CDR3) (23). The T_h cell determinant and the B cell epitope differ by only 2 amino acid residues (A → V and N → D) at positions 5 and 6, but this is sufficient to account for absolute specificity of the T cell response in C57Bl/6 mice (24).

Spleen cells were harvested 14 days after intraspleenic DNA inoculation, a time corresponding to the peak of the CD4 T cell response (24). In three independent experiments we found that all immunized *rel*B$^{-/-}$ BMC responded with activation of CD4 T cells specific for the T_h cell determinant (Table 2). Overall, the response in *rel*B$^{-/-}$ BMC was weaker than in unmanipulated C57Bl/6 mice, but comparable to C57Bl/6 BMC used as controls, reflecting possible effects by lethal irradiation/BM reconstitution. No proliferation was observed using spleen lymphocytes from a naive C5Bl/6 mouse, indicating that DNA immunization specifically primed naive T cells rather than expanding memory precursor T cells. The magnitude and specificity of the proliferative response by spleen lymphocytes were mirrored by the levels of IL-2 in the corresponding culture supernatants (Table 3). IL-2 was detected in the supernatants of all cultures irrespective of the experimental category. IFN-γ and IL-4 were also detected, albeit in different amounts, in agreement with the fact that somatic transgene immunization expands mainly uncommitted (T_h0) CD4 T cells (24). Thus, the deficit in BM-derived DC in *rel*B $^{-/-}$ BMC did not affect the magnitude or the type of CD4 T cell response following immunization via intraspleenic inoculation of DNA. From the foregoing, we conclude that following immunization by directly targeting the spleen as the site of immune induction, a specific CD4 T cell response can be induced in the absence of functional DC.

Table 2. CD4 T cell proliferative response in the spleen of *rel*B$^{-/-}$ BMC

Experiment	Strain	No. of mice	[^{3}H]Thymidine[a]	
			NANP	NVDP
I	C57Bl/6	4	1471 ± 853	14,322 ± 355
	*rel*B$^{-/-}$ BMC	4	1854 ± 906	17,432 ± 2052
II	C57Bl/6	2	182 ± 52	32,680 ± 5667
	C57Bl/6 BMC	4	768 ± 71	20,362 ± 5667
	*rel*B$^{-/-}$ BMC	4	421 ± 49	10,788 ± 1419
III	C57Bl/6 BMC	4	982 ± 81	5237 ± 1240
	*rel*B$^{-/-}$ BMC	4	2696 ± 788	16,142 ± 1874

[a]Results are expressed as mean c.p.m. of values of single mice each being tested in triplicate; c.p.m. of naive control C57Bl/6 mice cultured with the NVDP peptide = 588 ± 147.

Discussion

DC are considered a key player in T cell activation and are possibly the most potent regulators of the immune response (1). DC express a variety of surface receptors for the internalization of antigens (4) and go through the maturation process quite rapidly (30). *In vivo* clustering and interaction between DC and T cells have been visualized in draining LN, and found to be rapid (~24 h) (12). Immunization with protein antigen in Freund's' adjuvant showed that DC and not B cells present antigen to CD4 T cells in LN (31). However, after i.v. injection of soluble antigen, DC are only as good as B cells in capturing antigen and expressing MHC–peptide complex (13). Thus, whether DC or B cells serve as the APC for CD4 T cells seems to depend on the characteristics of the immunization (antigen depot in immunological adjuvant versus soluble antigen). Here we used *rel* B$^{-/-}$ BMC whose spleen DC, as demonstrated here, are unable to prime T cells to soluble antigen or processed peptide, to see whether T cell priming in

Table 3. Cytokine analysis in the spleen

Strain	No. of mice	IL-2 (ng/ml)	IL-4 (pg/ml)	IFN-γ (ng/ml)
*rel*B$^{-/-}$ BMC	4	357 ± 98	255 ± 78	124 ± 1
C57Bl/6 BMC	4	468 ± 71	564 ± 45	145 ± 23
C57Bl/6	2	865 ± 46	455 ± 36	164 ± 12

these mice is still possible following DNA immunization targeted to the spleen as the site of immune induction (22).

In the mouse, splenic DC of BM origin have been distinguished into two broad categories on the basis of surface expression of CD8α (32). These two phenotypically different categories have different localization in lymphoid tissues (2) and possibly different immunological functions (33). Homozygous *relB*$^{-/-}$ mice and *relB*$^{-/-}$ BMC (–/– → C57Bl/6) lack CD8a$^-$ DC, but have residual CD8α$^+$ DC (16). In our hands the absolute number of total splenic DC is low (Table 1). Moreover, these cells have a much-impaired ability to undergo maturation *in vitro* (Fig. 1). If compared with the findings of Wu *et al.* (16), our results show comparable expression of MHC class II molecules on residual spleen CD11c$^+$ cells of *relB* $^{-/-}$ BMC (Fig. 2). However, the severely compromised APC functions of residual spleen DC of *relB* $^{-/-}$ BMC documented here are in apparent contrast with the reported ability of these mice to induce an allo mixed lymphocyte reaction (16). There may be several reasons for this discrepancy. (i) Our BMC are generated using as recipients irradiated heterozygous *relB* (+/–) mice and not C57Bl/6 mice. (ii) We used an 85–95% pure DC population, whereas in the mixed lymphocyte culture experiment DC were purified by a different method and were used at a non-specified level of purity. (iii) More importantly, an allo mixed lymphocyte reaction reflects stimulation of T cells by DC, but does not imply processing and presentation of MHC antigens by self-MHC molecules. Therefore, while the two studies are not comparable, our findings establish that spleen DC of *relB*$^{-/-}$ BMC have a severely impaired APC function vis-à-vis nominal soluble antigen.

We documented multiple defects in APC function of residual spleen DC in *relB*$^{-/-}$ BMC. Injection of the FITC-conjugated OVA disclosed a markedly reduced ability to capture circulating soluble antigen *in vivo*. However, this alone cannot explain why *relB*$^{-/-}$ DC failed to present OVA after *in vitro* pulsing because this experiment was performed at a high antigen concentration (500 μg/ml). This also argues against a role of residual spleen DC during somatic transgene immunization where the concentration of the transgene product at peak time is ≤70 ng/ml (21), i.e. at least 4 × 10^3-fold less than in the *in vitro* pulsing experiment. As to their ability to present peptide antigen, our data show that residual spleen DC from *relB* $^{-/-}$ BMC cannot prime OVA-specific CD4 T cells unless used in high number. Even so, their antigen-presenting capacity is ~3-fold lower than that of *relB* competent spleen DC.

The experiments presented here are the first direct evidence that residual DC in *relB*$^{-/-}$ BMC are functionally defective, and *de facto* unable, to induce antigen-specific T cell responses. Whether the defect is intrinsic to the residual CD8a$^+$ subset or secondary to the lack of *relB*-competent CD8a$^-$ DC cannot be established. The fact that both DC types appear to originate from a common myeloid progenitor (34,35) and the *relB* $^{-/-}$ BM fails to generate functional DC in hemizygous recipients suggest that the immunological incompetence of CD8α$^+$ DC documented here may be part of the same genetic defect.

If residual DC cannot prime T cells, how are T cells primed following somatic transgene immunization? Several considerations point to a direct involvement of *in vivo* transgenic B lymphocytes. We have already documented that the transgene persists in spleen B lymphocytes for a protracted period of time (22). Notably, the Ig H transgene is under control of a B cell-specific promoter. Also, in our hands, spleen-derived DC are not transfected by the plasmid DNA used in the present study (our unpublished data). Finally, the amount of secreted transgenic product (≤70 ng/ml) is too low to enable uptake by *relB*$^{-/-}$ BMC DC and T cell priming. In addition, the possibility of T cell activation by cross-priming (36), albeit plausible, can be ruled out based on the fact that *relB*$^{-/-}$ BMC fail to induce a cytotoxic T lymphocyte response against a model tumor antigen via cross-priming (37). Taken together, the scenario we favor at this point is that after DNA injection into the spleen of *relB*$^{-/-}$ BMC, antigen synthesis, processing and presentation occur in B lymphocytes as the default pathway. Whatever the mechanisms might be, our results provide evidence that CD4 T cell priming in the spleen as the site of immune induction can occur in the absence of functional DC. While residual splenic DC of *relB*$^{-/-}$ BMC failed to prime T cells against the model antigen OVA *in vitro* and *in vivo*, specific T cell priming in these mice was demonstrated following somatic transgene immunization. Thus, transgenic B lymphocytes may be the key APC in our experiments since B lymphocytes can present their own Ig V region peptides to CD4 T cells *in vitro* (38) and the expansion of T cells *in vivo* can be driven by activated (39–41) or naive (42) B lymphocytes. Preliminary experiments show that that B lymphocytes transgenic for a plasmid coding for $OVA_{323-339}$ CD4 peptide can prime naive CD4 T cells from OT-II mice in the absence of DC (our unpublished data), strengthening the findings of this paper.

In conclusion, we demonstrate that CD4 T cell priming during an adaptive immune response originating in the spleen as the site of immune induction can occur without any manifest role for resident DC. Our findings are relevant to the mechanisms of antigen presentation during a systemic adaptive immune response where the initial interaction between antigen, B lymphocytes as the APC and T cells occurs in the spleen. While bringing new elements to our understanding on the function of DC and B cells in the local splenic environment, and their respective role in the initiation of T cell immunity, these findings begin to elucidate the role played by the *relB* gene in gauging the adaptive T cell response *in vivo* through its control of DC function.

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Abbreviations

APC	antigen-presenting cell
BM	bone marrow
BMC	bone marrow chimera
DC	dendritic cell
GM-CSF	granulocyte macrophage colony stimulating factor
OVA	ovalbumin

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my·e·lo·cyte (mī'ĕ-lō-sīt). **1.** A young cell of the granulocytic series, occurring normally in bone marrow, but not in circulating blood (except in certain diseases). When stained with the usual dyes, the cytoplasm is distinctly basophilic and relatively more abundant than in myeloblasts or promyelocytes, even though m.'s are smaller cells; numerous cytoplasmic granules (*i.e.*, neutrophilic, eosinophilic, or basophilic) are present in the more mature forms of m.'s, and the first two types are peroxidase-positive. The nuclear chromatin is coarser than that observed in myeloblasts, but it is relatively faintly stained and lacks a well defined membrane; the nucleus is fairly regular in contour (*i.e.*, not indented), and seems to be "buried" beneath the numerous cytoplasmic granules. **2.** A nerve cell of the gray matter of the brain or spinal cord. SYN medullocell. [myelo- + G. *kytos*, cell]

m. A, the youngest form of m., characterized by only a few (not more than ten) cytoplasmic granules, which are most reliably demonstrated by means of staining with neutral red; the mitochondria are numerous, and resemble those of the myeloblast.

m. B, the intermediate form of m., characterized by approximately 30 to 100 (or more) cytoplasmic granules scattered among the mitochondria; the latter are less numerous than in m.'s of the A stage, and they are frequently displaced toward the periphery of the cell.

m. C, the most mature of the m.'s characterized by numerous cytoplasmic granules that are recognizable as neutrophilic, eosinophilic, and basophilic; with neutral red these are stained, respectively, red, bright yellow, and deep maroon; C m.'s are frequently larger than earlier forms; if the nucleus is indented, the m. is maturing into a metamyelocyte.

my·e·lo·cy·the·mia (mī'ĕ-lō-sī-thē'mē-ă). The presence of myelocytes in the circulating blood, especially in persistently large numbers (as in myelocytic leukemia). [myelocyte + G. *haima*, blood]

my·e·lo·cyt·ic (mī'ĕ-lō-sit'ik). Pertaining to or characterized by myelocytes.

my·e·lo·cy·to·ma (mī'ĕ-lō-sī-tō'mă). A nodular focus or fairly well-circumscribed, relatively dense accumulation of myelocytes, as in certain tissues of persons with myelocytic leukemia. [myelocyte + G. *-oma*, tumor]

my·e·lo·cy·to·ma·to·sis (mī'ĕ-lō-sī'tō-mă-tō'sis). **1.** A form of tumor involving chiefly the myelocytes. SYN leukochloroma. **2.** A rare leukosis of fowl marked by the presence of white tumors composed of myeloid cells, located principally along the sternum and in the liver.

my·e·lo·cy·to·sis (mī'ĕ-lō-sī-tō'sis). The occurrence of abnormally large numbers of myelocytes in the circulating blood, or tissues, or both. [myelocyte + G. *-osis*, condition]

my·e·lo·di·as·ta·sis (mī'ĕ-lō-dī-as'tă-sis). Softening and destruction of the spinal cord. [myelo- + G. *diastasis*, separation]

my·e·lo·dys·pla·sia (mī'ĕ-lō-dis-plā'zē-ă). **1.** An abnormality in development of the spinal cord, especially the lower part of the cord. **2.** Inappropriate term for spina bifida occulta. [myelo- + G. *dys-*, difficult, + *plasis*, a molding]

my·e·lo·fi·bro·sis (mī'ĕ-lō-fī-brō'sis). Fibrosis of the bone marrow, especially generalized, associated with myeloid metaplasia of the spleen and other organs, leukoerythroblastic anemia, and thrombocytopenia, although the bone marrow often contains many megakaryocytes. SYN myelosclerosis, osteomyelofibrotic syndrome.

my·e·lo·gen·e·sis (mī'ĕ-lō-jen'ĕ-sis). **1.** Development of bone marrow. **2.** Development of the central nervous system. **3.** Formation of myelin around an axon.

my·e·lo·ge·net·ic, my·e·lo·gen·ic (mī'ĕ-lō-jĕ-net'ik, -jen'ik). **1.** Relating to myelogenesis. **2.** Produced by or originating in the bone marrow. SYN myelogenous.

my·e·log·e·nous (mī-ĕ-loj'ĕ-nŭs). SYN myelogenetic (2).

my·e·lo·gone, my·e·lo·go·ni·um (mī'ĕ-lō-gōn, mī'ĕ-lo-gō'nē-ŭm). An immature white blood cell of the myeloid series that is characterized by a relatively large, fairly deeply stained, finely reticulated nucleus that contains palely stained nucleoli, and a scant amount of rimlike, nongranular, moderately basophilic cytoplasm. M.'s are difficult to distinguish from lymphoblasts and monoblasts, unless one evaluates them in relation to the more mature forms usually associated with the younger cells. SYN myelogonium. [myelo- + G. *gonē*, seed]

my·e·lo·gram (mī'breve;e-lō-gram). Radiographic contrast study of the spinal subarachnoid space and its contents.

cervical m., contrast medium introduced directly into the cervical subarachnoid space, or moved with the help of gravity from the lumbar region, to outline the cervical cord and nerve roots.

lumbar m., most common study for herniated nucleus pulposus or intervertebral disc protrusion.

my·e·log·ra·phy (mī'ĕ-log'ră-fē). Radiography of the spinal cord and nerve roots after the injection of a contrast medium into the spinal subarachnoid space. [myelo- + G. *graphē*, a drawing]

my·e·lo·ic (mī-ĕ-lō'ik). Pertaining to the tissue and precursor cells from which neutrophils, eosinophils, and basophils are derived.

my·e·loid (mī'ĕ-loyd). **1.** Pertaining to, derived from, or manifesting certain features of the bone marrow. **2.** Sometimes used with reference to the spinal cord. **3.** Pertaining to certain characteristics of myelocytic forms, but not necessarily implying origin in the bone marrow. [myel- + -oid]

my·e·loi·do·sis (mī'ĕ-loy-dō'sis). General hyperplasia of myeloid tissue.

my·e·lo·leu·ke·mia (mī'ĕ-lō-lū-kē'mē-ă). A form of leukemia in which the abnormal cells are derived from myelopoietic tissue.

my·e·lo·li·po·ma (mī'ĕ-lō-li-pō'mă). A misnomer for certain nodular foci that are not neoplasms, but probably represent accumulations of cells derived from localized proliferation of reticuloendothelial tissue in the blood sinuses of the adrenal glands; grossly, the nodules may seem to be adipose tissue, but actually are foci of bone marrow containing erythropoietic or myeloid cells.

my·e·lo·lym·pho·cyte (mī'ĕ-lō-mon'ō-sīt). Rarely used term for an abnormal form of the lymphocytic series in the bone marrow, and presumed to be formed in that tissue.

my·e·lol·y·sis (mī-ĕ-lol'i-sis). Decomposition of myelin.

my·e·lo·ma (mī-ĕ-lō'mă). **1.** A tumor composed of cells derived from hemopoietic tissues of the bone marrow. **2.** A plasma cell tumor. [myelo- + G. *-oma*, tumor]

Bence Jones m., multiple m. in which the malignant plasma cells excrete only light chains of one type (either κ or λ); lytic bone lesions occur in about 60% of the cases, and light chains (Bence Jones protein) occur in the urine; amyloidosis and severe renal failure are more common than in multiple m. SYN L-chain disease, L-chain m.

endothelial m., SYN Ewing's *tumor*.

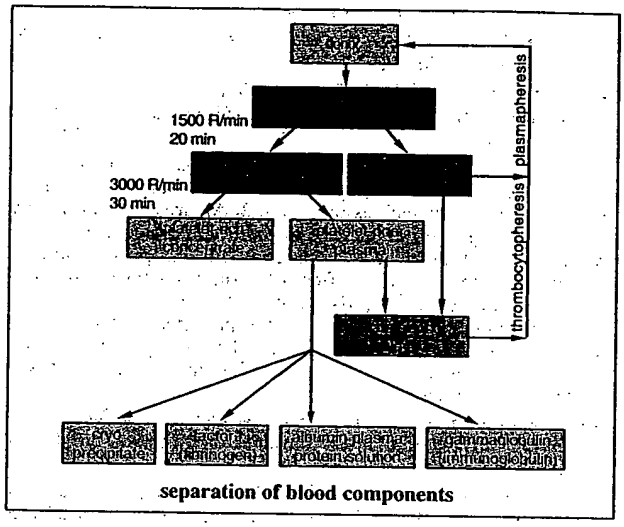
giant cell m., SYN giant cell *tumor* of bone.

L-chain m., SYN Bence Jones m.

multiple m., m. mul'tiplex, an uncommon disease that occurs more frequently in men than in women and is associated with anemia, hemorrhage, recurrent infections, and weakness. Ordinarily, it is regarded as a malignant neoplasm that originates in bone marrow and involves chiefly the skeleton, with clinical features attributable to the sites of involvement and to abnormalities in formation of plasma protein; characterized by numerous diffuse foci or nodular accumulations of abnormal or malignant plasma cells in the marrow of various bones (especially the skull), causing palpable swellings of the bones, and occasionally in extraskeletal sites; radiologically, the bone lesions have a characteristic punched-out appearance. The myeloma cells produce abnormal proteins in the serum and urine; those formed in any one example of multiple m. are different from other m. proteins, as well as from normal serum proteins, the most frequent abnormalities in the metabolism of protein being: 1) the occurrence of Bence Jones proteinuria, 2) a great increase in monoclonal γ-globulin in the plasma, 3) the occasional formation of cryoglobulin, and 4) a form of primary amyloidosis. The Bence Jones protein is not a derivative of abnormal serum protein, but seems to be formed *de novo* from amino acid precursors. SEE ALSO plasma cell m. SYN multiple myelomatosis, myelomatosis multiplex, plasma cell m. (1).

nonsecretory m., multiple m. in which there is no detectable paraproteinemia or paraproteinuria.

plasma cell m., **(1)** SYN multiple m. **(2)** plasmacytoma of bone,



muscle p., an alkaline fluid in muscle that is spontaneously coagulable, separating into myosin and muscle serum.

normal human p., sterile p. obtained by pooling approximately equal amounts of the liquid portion of citrated whole blood from eight or more adult humans who have been certified as free from any disease which is tranmissible by transfusion, and treating it with ultraviolet irradiation to destroy possible bacterial and viral contaminants.

salted p., the fluid portion of blood drawn from the vessels, which is prevented from coagulating by being drawn into a solution of sodium or magnesium sulfate. SYN salted serum.

△**plasma-, plasmat-, plasmato-, plasmo-.** Formative, organized; plasma. [G. *plasma*, something formed]

plas·ma·blast (plaz'mă-blast). Precursor of the plasma cell. SYN plasmacytoblast. [plasma + G. *blastos*, germ]

plas·ma cell dys·cra·sia. A diverse group of diseases characterized by the proliferation of a single clone of cells producing a monoclonal immunoglobulin or immunoglobulin fragment (a serum M component). The cells usually have plasma cell morphology, but may have lymphocytic or lymphoplasmacytic morphology. This group includes multiple myeloma, Waldenström's macroglobulinemia, the heavy chain disease, benign monoclonal gammopathy, and immunocytic amyloidosis.

plas·ma·crit (plaz'mă-krit). A measure of the percentage of the volume of blood occupied by plasma, in contrast to a hematocrit. [plasma + G. *krinō*, to separate]

plas·ma·cyte (plaz'mă-sīt). SYN plasma *cell*.

plas·ma·cy·to·blast (plas-mă-sī'tō-blast). SYN plasmablast.

plas·ma·cy·to·ma (plaz'mă-sī-tō'mă). A discrete, presumably solitary mass of neoplastic plasma cells in bone or in one of various extramedullary sites; in man, such lesions are probably the initial phase of developing plasma cell myeloma. [plasmacyte + G. *-oma*, tumor]

plas·ma·cy·to·sis (plaz'mă-sī-tō'sis). **1.** Presence of plasma cells in the circulating blood. **2.** Presence of unusually large proportions of plasma cells in the tissues or exudates. [plasmacyte + G. *-osis*, condition]

plas·ma ex·pand·er (plaz'mă eks-pan'der). SYN plasma *substitute*.

plas·ma·gene (plaz'mă-jēn). A determinant of an inherited character located in the cytoplasm. SYN cytogene. [plasma + gene]

plas·ma·ki·nins (plaz'mă-kīn'inz). A group of highly active oligopeptides found in sera that act upon smooth muscle of blood vessels, uterus, bronchi, etc.; *e.g.*, bradykinin, kallidin.

plas·ma·lem·ma (plaz-mă-lem'ă). SYN cell *membrane*. [plasma + G. *lemma*, husk]

plas·mal·o·gens (plaz-mal'ō-jenz). Generic term for glycerophospholipids in which the glycerol moiety bears a 1-alkenyl ether group (on rarer occasions, a 1-alkyl ether group); e.g., alk-1-enylglycerophospholipid; p. synthesis is reduced in disorders of the peroxisome. SYN phosphoglyceracetals.

blood plasma

selected components of plasma or serum, normal parameters

ammonia-N (whole blood)	53-143*	μmol/L
bicarbonate	21-25	mmol/L
bilirubin (total)	25.1-18.8*	μmol/L
bilirubin (direct)	up to 6.8	μmol/L
lead (whole blood)	up to 2.0	μmol/L
blood sugar: see glucose		
calcium	2.2-2.7	mmol/L
chloride	94-103	mmol/L
cholesterol	3.36-6.72	mmol/L
creatine	0-23-61	μmol/L
	0-23-92	
creatinine	0-62-106	μmol/L
	0-44-88	
iron	0-16-1-25	μmol/L
	0-14-3-31	
iron-binding capacity		
total	0-53-7-16	μmol/L
	0-44-8-62	
free	0-35-8-537	μmol/L
	0-26-9-44-8	
protein, total	67-87	g/L
fat, total	3-6-8-2	g/L
fatty acids, free	200-900	μmol/L
fructose	up to 0-55	mmol/L
galactose	up to 0-24	mmol/L
glucose	3-33-5-55	mmol/L
glycerine, total	0-27-2-88	mmol/L
glycerine, free	up to 0-25	mmol/L
uric acid	0-135-404	μmol/L
(enzymatic)	0-119-375	
urea	3-33-6-66	mmol/L
potassium	4-135-6*	mmol/L
copper	0-11-0-22-0	μmol/L
	0-13-4-24-4	g/L
β-lipoproteide	3-6-6-4	μmol/L
lithium	0-4-6-3	mmol/L
magnesium	0-66-0-90	mmol/L
lactate	1-00-1-78	mmol/L
sodium	137-148	mmol/L
phosphatide	1-74-3-94	mmol/L
inorganic phosphorus	0-81-1-65	mmol/L
thyroxine	66-187	nmol/L
triglyceride	0-97-2-70	mmol/L

*age-dependent

plas·mals (plaz'mălz). Long-chain aldehydes occurring in plasmalogens; *e.g.*, stearaldehyde, palmitaldehyde.

plas·ma·phe·re·sis (plaz'mă-fĕ-rē'sis). Removal of whole blood from the body, separation of its cellular elements by centrifugation, and reinfusion of them suspended in saline or some other plasma substitute, thus depleting the body's own plasma without depleting its cells. [plasma + G. *aphairesis*, a withdrawal]

plas·ma·phe·ret·ic (plaz'mă-fĕ-ret'ik). Relating to plasmapheresis.